Electrochemical SNP Detection Using Glucose Dehydrogenase
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ABSTRACT
Single nucleotide polymorphisms (SNPs) are important biomarkers for evaluating sensitivity to drugs and for predicting whether people might have a disease in the future. In this study, we constructed an electrochemical detection system of an SNP of peroxisome proliferator-activated receptor γ2 (PPARγ2) (C34G) using glucose dehydrogenase (GDH) based on a single base extension method. Target DNA was hybridized with capture DNA with 10^−8 mol dm^−3 detection limit based on amperometric sensing system.

Keywords : Single Nucleotide Polymorphism, Glucose Dehydrogenase, Single Base Extension

1. Introduction

Single nucleotide polymorphisms (SNPs) are attractive biomarkers for predicting whether people will have a disease in the future. Additionally, SNPs provide important clues for matching the right medicine to the right person, with the right dosage at the right time. Some SNPs are approved as biomarkers for diagnosis and selection of a suitable drug for each person by the U.S. Food and Drug Administration.

Many studies have developed SNPs genotyping systems as reviewed by Sobrino et al. Although hybridization based DNA sensors are attractive for discriminating DNA specifically, they require a specific design. On the other hand, an enzyme based assay can discriminate SNPs specifically based on enzyme substrate specificity and Patolsky et al. reported an attractive approach based on a single base extension method.

Figure 1 shows a scheme of SNP detection based on a single base extension method. DNA probes that are complementary DNA with downstream sequence of a mutation of target DNA are immobilized on a gold electrode. Target DNA hybridizes with immobilized probe DNA. Biotinylated dNTP base that is complementary with a mutant is extended on the probe DNA in the presence of DNA polymerase if the target DNA has a mutation. Then avidin conjugated with the enzyme (avidin GDH) binds to the DNA probe in which a biotin was inserted resulting in increase of current after washing of excess avidin-GDH and addition of glucose. In this system, PPARγ2 (C34G) was detected specifically with 10^−8 mol dm^−3 detection limit based on amperometric sensing system.

2. Experimental

2.1 Materials
Flavin adenine dinucleotide dependent GDH (FADGDH) was obtained by recombinant expression in Escherichia coli. 1-Methoxyphenazine methosulfate (m-PMS) and glutaraldehyde solution was purchased from Wako Chemicals. Bio-11-dCTP (Biotin-11-2′-deoxyctydine-5′-triphosphate) was purchased from Enzo Diagnostics, Inc. Gene Taq polymerase was purchased from Nippon gene.

2.2 Preparation of avidin conjugated glucose dehydrogenase
Avidin-FADGDH conjugate was prepared using glutaraldehyde crosslinking, as has been described elsewhere as well as avidin-
CATAGTCACTTCCTTAGGCAAAGAC
extension of b-dCTP on the DNA probe. Simultaneously, we observed a small difference of response current between incubation at 60°C. When all reagents were incubated simultaneously. High temperature would enhance the specificity of DNA extension and decrease DNA mishybridization and nonspecific binding. To shorten the sensing time, we tried to perform all steps in a single step. However, it required several hours to measure SNPs.

Figure 3 shows the results of measurement of PPAR-SNP-DNA. 10⁻⁶ M PPAR-SNP-DNA or PPAR-DNA was added to hybridize with immobilized probe DNA. 1 U avidin-FADGDH was added and incubated with the electrode for 20 min after extension of b-dCTP by Taq DNA polymerase for 10 min. The operational potential: 100 mV vs. Ag/AgCl. Temperature for measurement: 40°C. Each bar represents Δcurrent (nA) in the presence of 30 mM glucose.

Figure 2. Amperometric detection of PPAR-SNP-DNA. 10⁻⁶ M PPAR-SNP-DNA or PPAR-DNA was added to hybridize with immobilized probe DNA. 1 U avidin-FADGDH was added and incubated with the electrode for 20 min after extension of b-dCTP by Taq DNA polymerase for 10 min. The operational potential: 100 mV vs. Ag/AgCl. Temperature for measurement: 40°C. Each bar represents Δcurrent (nA) in the presence of 30 mM glucose.

3. Results and Discussion

3.1 Glucose dehydrogenase for labeling enzyme
To develop a highly sensitive detection system, the background signal should be decreased to reduce noise signals. Although we have already reported a DNA sensing system using Pyrroloquinoline quinone dependent GDH (PQQGDH) that has high catalytic activity (ca. 5000 U mg⁻¹ protein), it binds to DNA nonspecifically via cationic amino acids on its surface resulting in high background signals (data not shown). Then we selected bacterial FADGDH, which has a lower pI value (pI = 6.0) than PQQGDH (pI = 10.5).

We reported the cloning and characterization of thermostable bacterial FADGDH derived from Burkholderia cepacia. Although FADGDH has a lower catalytic activity (ca. 95 U mg⁻¹ protein) than PQQGDH, FADGDH maintained a sufficient catalytic activity for labeling enzyme after conjugation with avidin (maintained 78% activity compared with intact FADGDH).

3.2 Amperometric SNP sensing
The SNP in PPAR-DNA was detected by the single base extension method using avidin conjugated FADGDH. First of all, target DNA was incubated with the electrode followed by reaction of single base extension and incubation with avidin conjugated FADGDH. However, it required several hours to measure SNPs. To shorten the sensing time, we tried to perform all steps simultaneously. High temperature would enhance the specificity of extension and decrease DNA mishybridization and nonspecific binding of avidin-FADGDH. Since FADGDH that derived from B. cepacia has thermo stability, FADGDH activity is not lost during incubation at 60°C. When all reagents were incubated simultaneously, we observed a small difference of response current between PPAR-SNP-DNA and PPAR-DNA (data not shown). This indicates that b-dCTP would bind to avidin conjugated FADGDH before extension of b-dCTP on the DNA probe.

To avoid competing of extension of b-dCTP against binding of b-dCTP to avidin-FADGDH, avidin-FADGDH was added after 10 min extension reaction by Taq DNA polymerase. Figure 2 shows the results of SNP sensing. We observed a higher current in the presence of PPAR-SNP-DNA than in the presence of PPAR-DNA, control (40-mer randomized DNA) or without DNA. Since there was little difference between the presence of PPAR-DNA and in the absence of DNA, little b-dCTP was reacted with PPAR-DNA.

Then we investigated the dose dependency of this system. Figure 3 shows the results of measurement of PPAR-SNP-DNA and PPAR-DNA. Black circle represents response current of PPAR-SNP-DNA. Open circle represents response current of PPAR-DNA. Glucose concentration is 30 mM. Error bars indicate s.d. (n = 4).

Although there are many reports on detecting SNPs, a single base extension method using DNA polymerase would discriminate SNPs more clearly than hybridization. Additionally, the activity of glucose dehydrogenase can be easily measured using a commercially available electrochemical glucose sensing system. It would be an attractive enzyme for developing a commercially available SNP biosensor. However, in this study, we observed a higher background signal in the absence of DNA although we used glucose dehydrogenase with a low pl value to avoid nonspecific interaction. In order to develop a more highly sensitive SNP

POQGDH. The activity of the POQGDH-avidin conjugate was measured using 1 mM 2,6-dichlorophenolindophenol (DCIP), m-PMS, 20 mM glucose, and we defined 1 unit of GDH activity as the amount of enzyme required to reduce 1.0 µmol DCIP per min in the presence of glucose.

2.3 Amperometric SNP genotyping
A gold electrode was immersed into the phosphate buffered saline (50 µL) containing 1-nmol thiol-modified DNA probe (5'-SH-AGTGAATGGGAATTCGTGCCTTACTG-3') for 12 h or more and the DNA probe was immobilized onto the gold electrode. After washing of the electrode by water, the electrode was incubated with 1% blocking reagent for hybridization (Roche applied science) to reduce nonspecific binding of DNA. PPAR-SNP-DNA (5'-TCA-CATAGTCACTTCCTTAGGCAAAGAC-3') or PPAR-DNA (5'-TCATAGTCACTTCCTTAGGCAAAGAC-3') (100 pmol), biotinylated dCTP (b-dCTP) (100 pmol) and Gene Taq DNA polymerase (2 U) were incubated with the DNA probe immobilized on the electrode for 10 min at 60°C. Then avidin conjugated FADGDH (1 U) was added to the solution and incubated for 20 min at 60°C. Unbound avidin conjugated FADGDH was removed by washing the electrode with the buffer [10 mM MOPS, 150 mM NaCl, 1 mM EDTA buffer (pH 7.0)] including 0.2% Tween20 for three times. Various concentrations of glucose solutions were added to the electrode and the response current was measured at 40°C at a potential of 0.1 V vs. Ag/AgCl in the measurement buffer [10 mM MOPS buffer (pH 7.0) containing 6 mM m-PMS].

Figure 3. Calibration curve corresponding to changes of the electronic current upon detection of different concentrations of PPAR-SNP-DNA and PPAR-DNA. Black circle represents response current of PPAR-SNP-DNA. Open circle represents response current of PPAR-DNA. Glucose concentration is 30 mM. Error bars indicate s.d. (n = 4).
detection system without PCR amplification, optimization of the amount of immobilized probe DNA, blocking of electrode, selection of different GDH or improvement of GDH with protein engineering are required.

4. Conclusion

Since SNPs affect drug susceptibility and side effects, the development of a biosensor that can identify SNPs rapidly is required for selection of the right drug for the right person. We developed an amperometric SNPs detection system based on a single base extension method. We succeeded in discriminating SNP of PPARγ2 with 10⁻⁸ M DNA as the detection limit. Amperometric sensing systems are attractive for simple measurement with a miniaturized biosensing system. Although we need to optimize the detection system since the detection limit of this study was insufficient without PCR amplification, we were able to construct an electrochemical SNP detection system.

References